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New withanolides from *Physalis minima* and their cytotoxicity against A375 human melanoma cells†

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Seven previously undescribed withanolides, namely physaminilide A–G (**1–7**), and two artificial withanolides (**8–9**), along with 10 known analogues (**10–19**) were isolated from *Physalis minima*. The structures were established by spectroscopic analysis, including NMR and electronic circular dichroism (ECD) data. Cytotoxicity of all the isolates was evaluated against A375 human melanoma cells. Compounds **2**, **5**, **8**, **10**, **11** and **15** exhibited significant cytotoxic activities with IC_{50} values in the range of 1.2–9.4 μM.

Introduction

Withanolides are a group of C_{28} steroidal lactones based on the ergostane skeleton that are common in the family Solanaceae.[1] Over 900 withanolides have been encountered to date,[2] and have captured the attention of researchers due to their diverse structures and pharmacological effects, including antitumor,[3–7] antimicrobial,[8] anti-inflammatory,[9–11] and immunosuppressive activities.[12]

The genus *Physalis* (Solanaceae), containing about 120 species worldwide, is known to produce a series of withanolides, showed significant antibacterial, anti-inflammatory, and antitumor effects. Five species and two varieties are distributed in mainland China.[13,14] *Physalis minima* L., an annual herb, has been used as a traditional folk medicine for various purposes,[15] and it is also utilized to treat analogous conditions in other countries, including Indian, Pakistan, and Japan. Various bioactive withanolides have been isolated from this plant.[16–21]

As part of a continuing research program on the genus *Physalis* to discover new withanolides with potential anticancer activities,[22–25] the chemical constituents of *P. minima* were investigated. Seven previously undescribed withanolides (**1–7**; Fig. 1), two artificial withanolides (**8–9**; Fig. 1), and 10 known compounds (**10–19**; Fig. 1) were isolated from a crude EtOAc extract of the aerial parts of this plant. The structures of **1–9** were established by 1D and 2D NMR and electronic circular dichroism (ECD) spectroscopic duly analyses. In addition, all of the isolated withanolides were evaluated for their cytotoxic activities against A375 human melanoma cells.

Results and discussion

Compound **1** (physaminilide A) was obtained as a white powder. Its molecular formula was determined to be $C_{30}H_{38}O_9$ on the basis of the ^{13}C NMR data and the positive ion peak at *m*/*z* 543.2583 ([M + H]$^+$, calcd for $C_{30}H_{39}O_9$, 543.2594) in the HRESIMS, which indicated 12 degrees of unsaturation. Analysis of the NMR data (Tables 1 and 3) clearly indicated that **1** is closely related to the known compound physagulide C,[26] with the major differences being due to several signals in the side chain. The ^1H NMR spectrum revealed the characteristic signals of H-2, H-3, H-4 and H-6 for a 1-oxo-2-ene-5β,6β-epoxy-4β-hydroxy-moiety at δ_H 6.53 (1H, d, J = 9.8 Hz), 7.32 (1H, dd, J = 9.8, 6.1 Hz), 4.08 (1H, d, J = 6.1 Hz) and 3.38 (1H, br s), respectively. The substitution pattern of rings A/B was confirmed by the signals at δ_C 203.2 (C-1), 132.2 (C-2), 146.1 (C-3), 70.7 (C-4), 64.4 (C-5) and 61.3 (C-6) in the ^{13}C NMR spectrum. The ^1H NMR and HSQC spectrum revealed that the signals at δ_H 6.65 (1H, br s) and 6.32 (1H, br s) could be assigned to the terminal double bond carbon at δ_C 125.7. Five methyl group signals [δ_H 1.35 (3H, s), 1.87 (3H, s), 1.22 (3H, d, J = 7.0 Hz), 1.55 (3H, s), and 2.01 (3H, s)] were observed for δ_C 16.7 (C-18), 17.3 (C-19), 18.3 (C-21), 29.3 (C-28), and 21.6 (-OAc), respectively. The degrees of unsaturation and the chemical shifts of C-24 (δ_C 69.7), C-25 (δ_C 146.5) and C-27 (δ_C 125.7) suggested that **1** possesses a six-membered double bond migration δ-lactone moiety in side chain, with the $\Delta^{24,25}$ double bond shifted to $\Delta^{25,27}$, and a hydroxy group linked to C-24.[27] These deductions were supported by the HMBC correlations from CH_3-28 (δ_H 1.55) to C-23/C-24/C-25, H-27a (δ_H 6.65) to C-24/C-25/C-26, H-27b (δ_H 6.32) to C-24/C-26, H-23a (δ_H 2.36) to C-22/C-24/C-28, and H-23b (δ_H 2.27) to C-24/C-25/C-28, and ^1H–^1H COSY correlations between H-22 (δ_H 4.63) and H-23a (δ_H

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† Electronic supplementary information (ESI) available: UV, IR, CD, HRESIMS and NMR spectra of **1–9**. See DOI: 10.1039/d0ra04106h

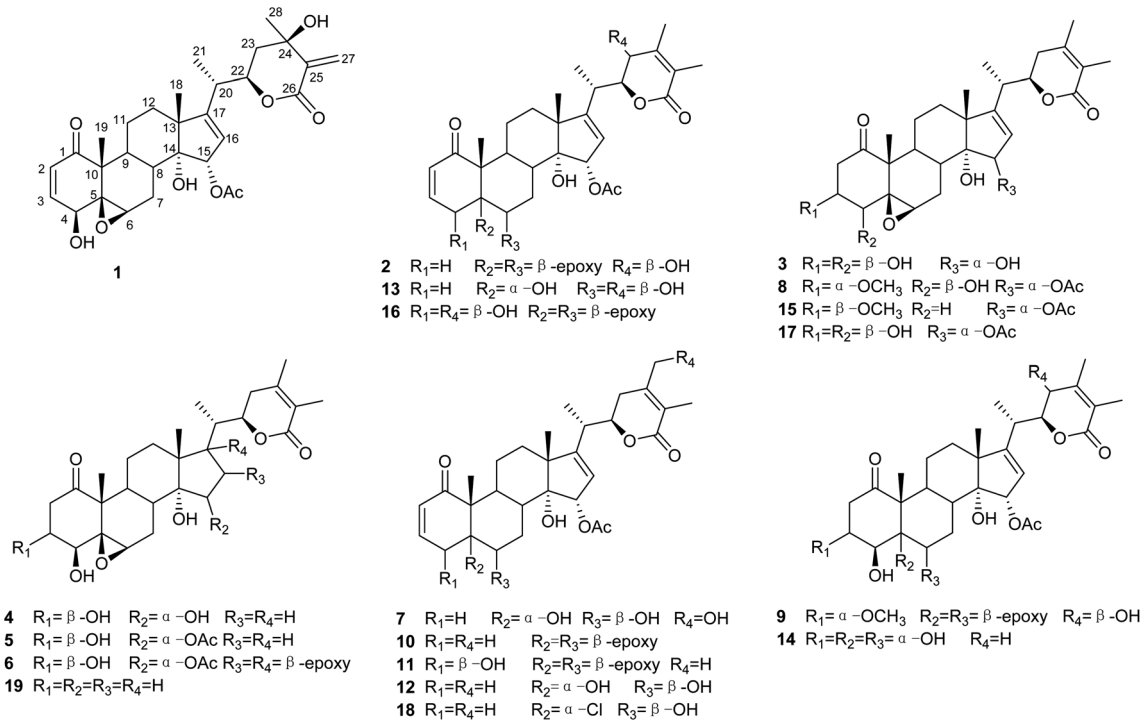

Fig. 1 Structures of compounds **1–19** from *Physalis minima*.

Table 1 ^{1}H NMR data of compounds **1–5** (pyridine-d_5, 600 MHz, δ in ppm, J in Hz)[a]

Position	1	2	3	4	5
2	6.53 d (9.8)	6.19 dd (10.0, 2.7)	3.31 dd (15.0, 7.5)	3.28 dd (15.0, 7.5)	3.43 dd (15.7, 7.5)
			3.28 dd (15.0, 2.5)	3.24 br d (15.0)	3.09 br dd (15.7)
3	7.32 dd (9.8, 6.1)	6.83 ddd (10.0, 6.0, 2.2)	4.72 m	4.70 m	4.72 m
4	4.08 d (6.1)	3.00 br d (19.0)	4.05 br s	4.03 br s	3.99 s
		1.95 br s			
6	3.38 br s	3.23 br s	3.89 br s	3.88 br s	3.71 s
7	2.94 m	2.84 br d (14.5)	3.24 m	3.13 m	3.02 br d (13.4)
	1.99 m	1.93 m	3.14 m	2.93 m	2.12 m
8	2.37 m	2.40 m	2.43 m	2.34 m	2.31 m
9	2.06 m	2.39 m	2.91 m	2.73 m	2.30 m
11	2.22 m	2.41 m	1.87 m	1.86 m	1.74 m
	2.04 m	1.55 m	1.64 m	1.67 m	1.58 m
12	1.79 m	1.84 m	2.11 m	2.61 br t (13.0)	2.10 m
	1.62 m	1.78 m	1.82 m	1.82 m	1.74 m
15	5.82 d (2.5)	5.88 d (2.6)	5.05 m	4.64 br s	5.57 br s
16	6.09 d (2.5)	6.39 d (2.6)	6.13 m	2.36 m	2.41 m
				1.91 m	1.96 m
18	1.35 s	1.47 s	1.35 s	1.37 s	1.34 s
19	1.87 s	1.37 s	1.85 s	1.85 s	1.75 s
20	2.64 m	3.13 m	2.63 m	2.37 m	2.32 m
21	1.22 d (7.0)	1.36 d (7.0)	1.27 d (7.0)	1.12 d (7.0)	1.09 d (7.0)
22	4.63 dt (12.0, 3.4)	4.47 br s	4.43 br dd (13.0, 3.0)	4.55 br dd (13.0, 3.0)	4.54 dd (13.0, 3.0)
23	2.36 m	4.46 br s	2.48 m	2.29 m	2.31 m
	2.27 m		2.07 m	1.88 m	1.96 m
27	6.65 br s	1.85 s	1.84 s	1.91 s	1.93 s
	6.32 br s				
28	1.55 s	1.82 s	1.50 s	1.60 s	1.60 s
OAc	2.01 s	2.00 s			2.21 s

[a] Assignments based on HSQC, HMBC, and NOESY data.

Table 2 ^{1}H NMR data of compounds **6–9** (pyridine-d_5, 600 MHz, δ in ppm, J in Hz)[a]

Position	6	7	8	9
2	3.45 dd (16.0, 7.2) 3.04 dd (16.0, 2.5)	6.17 dd (10.0, 2.5)	3.26 dd (16.0, 8.0) 2.96 m	3.25 dd (16.0, 8.0) 2.95 dd (16.0, 3.0)
3	4.70 m	6.70 ddd (10.0, 5.0, 2.1)	3.96 m	3.95 br dd (8.0, 3.0)
4	3.97 br s	3.78 dt (19.7, 2.5) 2.40 dd (19.7, 5.0)	3.94 m	3.93 br s
6	3.64 br s	4.21 br s	3.51 br s	3.49 br s
7	2.91 br d (14.3) 1.95 m	2.81 m 2.68 m	2.98 m 2.12 m	2.97 m 2.10 m
8	2.03 m	2.77 m	2.28 m	2.28 m
9	2.25 m	3.43 m	2.26 m	2.24 m
11	1.78 m 1.43 m	2.71 m 1.57 m	1.69 m 1.48 m	1.69 m 1.49 m
12	1.67 br d (13.3) 1.60 br d (13.3)	2.07 m 1.94 m	1.75 m 1.58 m	1.80 m 1.63 m
15	5.43 br s	6.21 d (2.6)	5.89 d (2.5)	5.93 d (2.6)
16	3.80 br s	6.02 d (2.6)	6.12 d (2.5)	6.41 d (2.6)
18	1.31 s	1.44 s	1.32 s	1.44 s
19	1.70 s	1.70 s	1.70 s	1.66 s
20	2.55 m	2.69 m	2.62 m	3.13 dd (14.0, 7.0)
21	1.00 d (7.0)	1.28 d (7.0)	1.23 d (7.0)	1.36 d (7.0)
22	4.49 br dd (12.6, 3.5)	4.50 dt (13.0, 3.9)	4.43 dt (13.0, 3.7)	4.48 br s
23	2.30 m 2.13 m	2.87 m 2.63 m	2.42 m 2.01 m	4.48 br s
27	1.90 s	1.93 s	1.85 s	1.85 s
28	1.73 s	4.45 d (14.6) 4.34 d (14.6)	1.51 s	1.84 s
OAc	2.32 s	2.18 s	2.21 s	2.19 s
OMe			3.30 s	3.29 s

[a] Assignments based on HSQC, HMBC, and NOESY data.

2.36)/H-23b (δ_H 2.27)/H-20 (δ_H 2.64) (Fig. 2). Since the NMR signals of rings B-D of compound **1** were in good agreement with those of physagulide C, the relative stereochemistry of **1** was supposed to be the same as physagulide C. Therefore, the hydroxy group linked to C-14 was proposed as α-oriented. The NOESY correlations of H-7a (δ_H 2.94) with H-8 (δ_H 2.37) and H-15 (δ_H 5.82) confirmed that the acetoxy group is α-oriented, and these of H-6 (δ_H 3.38) with H-4 (δ_H 4.08) and H-7b (δ_H 1.99) consistent with the hydroxy group at C-4 and 5,6-epoxy moiety being β-oriented. The NOESY correlations of the CH_3-28 (δ_H 1.55) with H-22 (δ_H 4.63) and H-23b (δ_H 2.27), and H-23b (δ_H 2.27) with H-22 (δ_H 4.63) implied that the hydroxy group at C-24 is β-oriented (Fig. 2).[28] The 20*S*,22*R* configurations of **1** were determined based on the biogenetic considerations and the characteristic small coupling pattern (3.4 Hz) of two gauche conformation protons, H-20 and H-22.[3] The absolute configuration of C-22 was further established as *R* based on the ECD positive Cotton effect at 250 nm.[25] Thus, the structure of **1** was assigned as (20*S*,22*R*)-15α-acetoxy-5β,6β-epoxy-4β,14α,24β-trihydroxy-1-oxowitha-2,16,25-trienolide, and has been named physaminilide A.

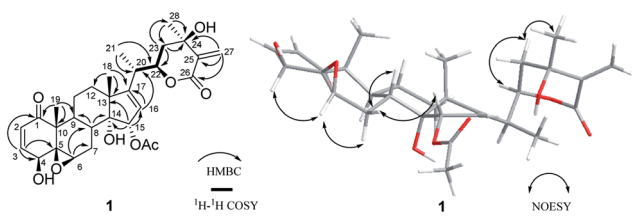

Fig. 2 Key ^{1}H–^{1}H COSY, HMBC and NOESY correlations for compounds **1**.

Compound **2** (physaminilide B) was assigned the molecular formula, $C_{30}H_{38}O_8$, based on its HRESIMS (*m*/*z* 527.2623 [M + H]$^+$, calcd for $C_{30}H_{39}O_8$, 527.2645) and ^{13}C NMR data. Detailed comparison of the NMR data of **2** (Tables 1 and 3) with those of physagulide D (**16**)[26] showed these compounds to share the same substitution pattern in rings B–D and the side chain. The upfield shift of C-4 (−37.2 ppm) at δ_C 33.6, and the assignment of δ_H 3.00 (1H, br d, J = 19.0 Hz), δ_H 1.95 (1H, br s) to H_2-4, suggested the absence of a hydroxy group at C-4 in **2**, which was corroborated by the HMBC correlations from H-2 to C-4/C-5, and H-4a to C-2/C-3/C-5/C-6 (Fig. 3). The NOESY correlations of H-8 with H-15 confirmed an α-orientation of the acetoxy group on C-15 (Fig. 4). Based on the positive Cotton effect at 250 nm in the ECD spectrum, a 22*R* configuration was suggested.[25] Thus, the structure of **2** was proposed as (20*S*,22*R*)-15α-acetoxy-5β,6β-epoxy-14α,23β-dihydroxy-1-oxowitha-2,16,24-trienolide.

The molecular formula of **3** (physaminilide C) was determined as $C_{28}H_{38}O_8$ from its ^{13}C NMR and HRESIMS (*m*/*z* 503.2647 [M + H]$^+$, calcd for $C_{28}H_{39}O_8$, 503.2645) data. Analysis of the NMR spectra suggested the structure of **3** (Tables 1 and 3) to be closely related to that of physaminimin F (**17**),[29] except for

the replacement of an acetoxy group at C-15 by a hydroxy group. The HMBC correlations from H-16 to C-13/C-14/C-15/C-17 were consistent with the location of a hydroxy group at C-15 (Fig. 3). The NOESY correlations of H-4 with H-3/H-6, H-7a (δ_H 3.24) with H-6/H-9, and H-15 with H-7b (δ_H 3.14) confirmed that the hydroxy group at C-15 is α-oriented, the hydroxy groups at C-3, C-4 are β-oriented, and the 5,6-epoxy moiety is β-oriented (Fig. 4). The positive Cotton effect at 250 nm in the ECD spectrum indicated a 22*R* configuration.[25] Thus, compound **3** was established as (20*S*,22*R*)-5β,6β-epoxy-3β,4β,14α,15α-tetrahydroxy-1-oxowitha-16,24-dienolide.

The molecular formula of **4** (physaminilide D) was determined as $C_{28}H_{40}O_8$ on the basis of the HRESIMS (*m*/*z* 505.2800 $[M + H]^+$, calcd for $C_{28}H_{41}O_8$, 505.2801) and ^{13}C NMR data. The NMR spectra of **4** (Tables 1 and 3) were closely comparable to those of compound **3**, except for the lack of any signals for a 16,17-double bond. This was corroborated by the HMBC correlations from CH_3-18 to C-17, and CH_3-21 to C-17 (Fig. 3). The NOESY correlations of H-15 with H-8/CH_3-18, confirmed that the hydroxy group at C-15 is α-oriented (Fig. 4). The orientation of 14-OH was proposed as α-oriented by the similarity of the NMR signals to those of **3**. The absolute configuration of C-22 was established as *R* through the ECD Cotton effect observed at 250 nm.[25] Thus, compound **4** was assigned as (20*S*,22*R*)-5β,6β-epoxy-3β,4β,14α,15α-tetrahydroxy-1-oxowitha-24-enolide.

Compound **5** (physaminilide E) gave a molecular formula of $C_{30}H_{42}O_9$ from its HRESIMS (*m*/*z* 547.29332 $[M + H]^+$, calcd for 547.2907) and ^{13}C NMR data. Analysis of the NMR data of **5** (Tables 1 and 3) showed close correlations to the data for physaminimin F (**17**).[29] The main difference was the absence of a 16,17-double bond in **6**. This was corroborated by the HMBC correlations from H-16a (δ_H 1.96) to C-13/C-14/C-15, CH_3-18 to C-17, and CH_3-21 to C-17 (Fig. 3). The α-orientation of OAc-15 was supported by the NOESY correlations H-7a (δ_H 3.02) with H-15/H-8 (Fig. 4). The absolute configuration of C-22 was established as *R* through the ECD Cotton effect observed at 250 nm.[25] Accordingly, compound **5** was established as (20*S*,22*R*)-15α-acetoxy-5β,6β-epoxy-3β,4β,14α-trihydroxy-1-oxowitha-16,24-dienolide.

Compound **6** (physaminilide F) gave a molecular formula of $C_{30}H_{40}O_{10}$ from its HRESIMS (*m*/*z* 561.2686 $[M + H]^+$, $C_{30}H_{41}O_{10}$, calcd for 561.2700) and ^{13}C NMR data. Comparison of the NMR data of **7** (Tables 2 and 3) with those of physaminimin F (**17**)[29]

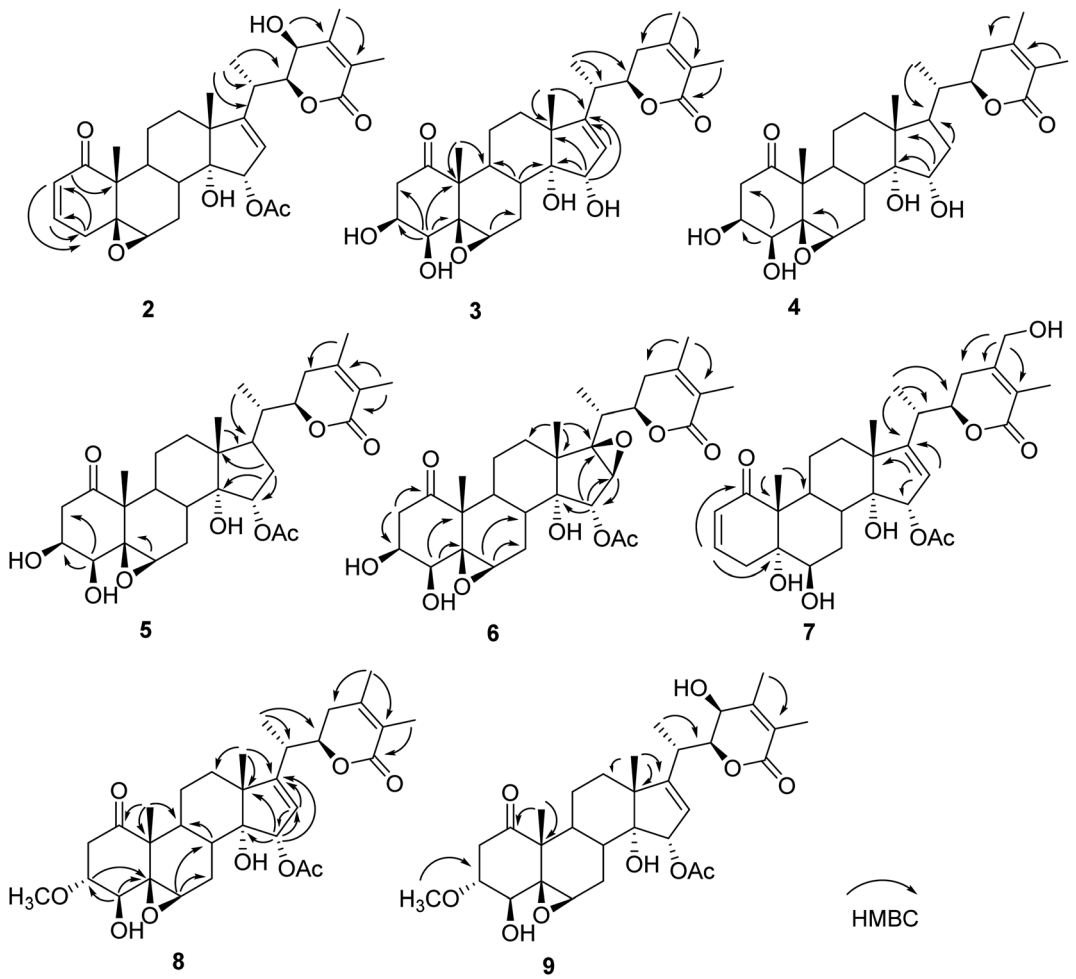

Fig. 3 Key HMBC correlations for compounds **2**–**9**.

indicated their identical A–C rings and the side chain. The key differences arose from the downfield shifts of C-16 (δ_C 59.4) and C-17 (δ_C 76.7) in **6** suggested the presence of a 16,17-epoxy group, which were confirmed by the HMBC correlations from H-15 to C-13/C-14/C-16/C-17, H-16 to C-14/C-15, and CH_3-18 to C-12/C-13/C-14/C-17 (Fig. 3). Its β-orientation was established by the NOESY correlations of H-16 with CH_3-21, H-12a (δ_H 1.67) with H-16/H-9 (Fig. 4). The absolute configuration of C-22 was established as *R* through the ECD Cotton effect observed at 250 nm.[25] Thus, the structure of **6** was established as (20*S*,22*R*)-15α-acetoxy-5β,6β:16β,17β-diepoxy-3β,4β,14α-trihydroxy-1-oxowitha-24-enolide.

Compound **7** (physaminilide G) was isolated as a white powder. Its molecular formula was determined to be $C_{30}H_{40}O_9$ based on the positive HRESIMS data at *m*/*z* 545.2723 $[M + H]^+$ (calcd for $C_{30}H_{41}O_9$, 545.2751) combined with the ^{13}C NMR data.

Its ^1H and ^{13}C NMR data (Tables 2 and 3) were similar to those of the known compound withaminimin (**12**),[30] with the only major difference being a set of oxygenated methylene protons observed for **7** at δ_H 4.34 (1H, d, $J = 14.6$ Hz) and δ_H 4.45 (1H, d, $J = 14.6$ Hz), which were assigned to a hydroxymethyl substituent at C-28 based on the HMBC correlations of H-28 (δ_H 4.34, δ_H 4.45) with C-23/C-24/C-25 (Fig. 3). From all the evidence obtained, compound **7** was determined as (20*S*,22*R*)-15α-acetoxy-5α,6β,14α,28-tetrahydroxy-1-oxowitha-2,16,24-trienolide.

Compound **8** (3-methoxy-2,3-dihydrowithangulatin A) gave a molecular formula of $C_{31}H_{42}O_9$ from the HRESIMS (*m*/*z* 559.2891 $[M + H]^+$, $C_{31}H_{43}O_9$, calcd for 559.2907) and ^{13}C NMR data. The NMR spectroscopic data of **8** (Tables 2 and 3) showed a close similarity with those of physaminimin F (**17**),[29] except for the presence of additional signals at δ_H 3.96 (1H, m) and δ_H 3.30 (3H, s), indicating the presence of a methoxy group in **8**. The HMBC correlations between OCH_3-3 [δ_H 3.30 (3H, s)] and C-3 (δ_C 78.9), suggested that the methoxy group was located at C-3 (Fig. 3). The NOESY correlations of H-3 with CH_3-19, H-16 with H-15/CH_3-18, H-6 with H-4/H-7a (δ_H 2.98), and H-7a with H-9 confirmed that the acetoxy group is α-oriented, the methoxy group at C-3 is α-oriented, and the hydroxy group at C-4 and the 5,6-epoxy moiety are β-oriented (Fig. 4). The ECD spectrum exhibited a positive Cotton effect at 250 nm, suggesting a 22*R* configuration.[25] Based on the above evidence, the structure of **8** was established as (20*S*,22*R*)-15α-acetoxy-5β,6β-epoxy-4β,14α-dihydroxy-3α-methoxy-1-oxowitha-16,24-dienolide.

The HRESIMS analysis of compound **9** (3-methoxy-2,3-dihydrophysagulide D) provided the molecular formula of $C_{31}H_{42}O_{10}$ (*m*/*z* 575.2850 $[M + H]^+$, calcd for $C_{31}H_{43}O_{10}$, 575.2856). The NMR spectra suggested that the structure of **9** (Tables 2 and 3) is closely related to that of physagulide D,[26] except for the absence of two olefinic protons of the α,β-unsaturated ketone in ring A, and the presence of signals at δ_H 3.95 (1H, br dd, $J = 8.0$, 3.0 Hz), δ_H 3.29 (3H, s) attributable to H-3 and OCH_3-3 in **9**. The methoxy

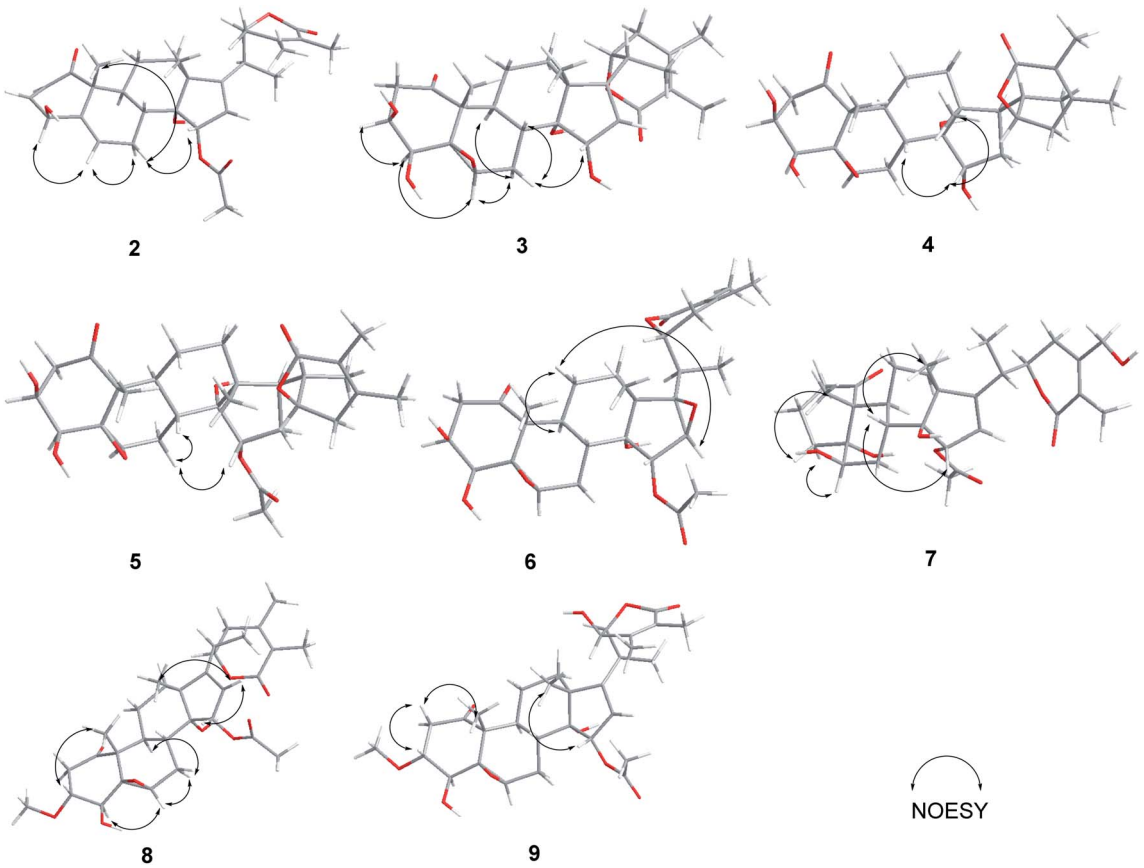

Fig. 4 Key NOESY correlations for compounds **2**–**9**.

Table 3 ^{13}C NMR data of compounds **1–9** (pyridine-d_5, 150 MHz, δ in ppm)[a]

Position	1	2	3	4	5	6	7	8	9
1	203.2	204.1	211.2	211.2	210.9	211.1	205.5	209.9	209.8
2	132.2	129.2	44.4	44.4	44.3	44.1	129.5	41.4	41.4
3	146.1	146.2	70.1	70.0	69.3	69.1	142.7	78.9	78.9
4	70.7	33.6	79.5	79.6	78.8	78.5	37.3	74.5	74.6
5	64.4	62.2	65.3	65.2	65.1	64.8	77.7	64.8	64.7
6	61.3	64.2	60.7	60.6	60.3	60.3	75.3	59.3	59.3
7	25.7	25.9	25.7	27.5	27.2	26.5	28.9	25.6	25.5
8	36.8	36.6	36.6	37.5	36.9	36.1	37.9	36.6	36.5
9	40.9	40.5	39.5	40.4	39.7	38.4	37.0	39.0	39.0
10	48.8	49.1	51.6	51.8	51.8	51.3	53.0	51.3	51.3
11	21.8	24.5	21.9	21.9	21.7	20.3	24.7	21.4	21.5
12	38.0	39.2	38.2	42.4	41.7	31.8	39.7	37.8	38.6
13	53.2	53.4	52.8	46.5	47.3	47.2	53.7	53.3	53.6
14	81.8	81.4	82.2	85.1	84.8	82.2	82.4	81.9	81.9
15	84.7	84.9	82.8	78.8	81.7	78.5	83.8	84.8	84.9
16	122.3	123.5	127.6	38.3	35.1	59.4	122.6	122.7	123.4
17	162.5	162.6	157.9	52.9	52.8	76.7	162.7	162.5	162.7
18	16.7	17.5	17.0	15.8	18.3	15.7	17.6	16.6	17.3
19	17.3	15.7	15.8	18.4	15.6	15.7	15.8	15.5	15.4
20	36.6	32.8	35.5	38.6	38.6	34.6	35.8	35.6	32.8
21	18.3	20.9	18.4	15.5	15.7	14.4	18.6	18.2	20.9
22	79.6	85.4	79.6	79.2	78.9	77.3	80.4	79.1	85.4
23	41.1	67.0	32.8	30.5	30.9	33.3	28.2	32.7	67.0
24	69.7	154.3	150.0	149.9	149.9	149.7	153.7	149.9	154.3
25	146.5	121.7	122.0	122.1	122.1	122.2	121.3	122.1	121.8
26	165.9	165.2	166.8	167.1	166.9	166.4	166.9	166.6	165.2
27	125.7	13.5	13.0	13.0	13.0	13.0	12.5	12.9	13.5
28	29.3	16.1	20.1	20.4	20.3	20.4	61.2	20.5	16.1
OAc	170.2	170.3			170.5	170.4	171.2	170.5	170.4
OAc	21.6	21.6			21.9	21.4	21.9	21.6	21.6
OMe								57.2	57.2

[a] Assignments based on HSQC, HMBC, and NOESY data.

group was determined to be located at C-3 (δ_C 78.9) by HMBC correlations between OCH_3-3 and C-3 (Fig. 3). The NOESY correlations of H-2a (δ_H 3.25) with H-3/CH_3-19, CH_3-18 with H-15, H-6 with H-4/H-7a (δ_H 2.97), and H-7a with H-9 confirmed that the acetoxy group at C-15 and the methoxy group at C-3 are α-oriented, the hydroxy group at C-4 and the 5,6-epoxy moiety are β-oriented (Fig. 4). The positive Cotton effect at 250 nm in ECD spectrum indicated a 22*R* configuration.[25] Therefore, **9** was identified as (20*S*,22*R*)-15α-acetoxy-5β,6β-epoxy-4β,14α,23β-trihydroxy-3α-methoxy-1-oxowitha-16,24-dienolide.

Compounds **8–9** were hypothesized to be intermolecular Michael-type addition adducts of polar protic solvent nucleophiles like methanol or ethanol,[31,32] the former solvent established compound **8**, named as 3-methoxy-2,3-dihydrowithangulatin A, while the latter solvent gave compound **9**, obtained as 3-methoxy-2,3-dihydrophysagulide D. Compound **11** (10 mg) was heated with methanol (5 mL) for 8 h to afford compound **8**. The methanol solution of compound **11** was examined at four time intervals (0, 2, 4, and 8 h) by HPLC analysis. The results showed the artifact **8** was increased in a time-dependent manner (Fig. S74†). Therefore, the use of methanol or ethanol should be cautious in the extraction and purification processes of withanolides with the 1-oxo-2-ene system in ring A.

By comparing their analytical data with those reported in the literature, the known compounds were identified as physagulin A (**10**),[33] withangulatin A (**11**),[34] withaminimin (**12**),[30] physagulin M (**13**),[33] physaliolide C (**14**),[11] physagulin N (**15**),[33] physagulide D (**16**),[26] physaminimin F (**17**),[29] physagulin B (**18**),[35] 27-deoxy-14-hydroxywithaferin A (**19**).[36]

Cytotoxicity of all the isolates was evaluated against A375 human melanoma cells. Among them, compounds **1**, **3–4**, **6–7**, **9**, **12–14**, and **16–19** were inactive for A375 human melanoma cells used (IC_{50} > 10 μM). Meanwhile, Compounds **2**, **5**, **8**, **10**, **11** and **15** exhibited significant cytotoxic activities with IC_{50} values in the range of 1.2–9.4 μM. These results indicated that withanolides with 5β,6β-epoxy possessed significant cytotoxic activities. The 16,17-double bond in ring D was essential for cytotoxicity. Furthermore, the 15-acetoxy group also had a slight influence on their cytotoxicity.

Experimental

General experimental procedures

Optical rotations were measured with a PerkinElmer 241 polarimeter. UV spectra were collected in a Shimadzu UV 2201 spectrophotometer. ECD spectra were recorded on a Bio-Logic Science MOS-450 spectrometer. IR spectra were obtained on

a Bruker IFS 55 spectrometer. NMR experiments were performed on Bruker AV-400 and AV-600 spectrometers. Chemical shifts are reported as δ (ppm) related to the solvent pyridine-d_5 (δ_H 7.58 and δ_C 135.91) as references, and coupling constants (J values) are given in Hz. HRESIMS data were obtained on an Agilent 6210 TOF mass spectrometer. Silica gel GF_{254}, obtained from Qingdao Marine Chemical Factory, was used for TLC. Sephadex LH-20 for gel-permeation chromatography was obtained from Pharmacia. Column chromatography (CC) was performed on silica gel (200–300 mesh, Qingdao Marine Chemical Factory) and octadecyl silica gel (Merck Chemical Company Ltd., Darmstadt, Germany). RP-HPLC separations were conducted using an LC-6AD liquid chromatograph, SPD-20A UV detector (Shimadzu, Kyoto, Japan), equipped with a YMC Pack ODS-A column (250 × 20 mm, 120 Å, 5 μm).

Plant material

Physalis minima was collected from Fujian Province, China, in September 2014, and identified by Professor Jincai Lu, Shenyang Pharmaceutical University. A voucher specimen (PM-2014) has been deposited in the herbarium of Shenyang Pharmaceutical University.

Extraction and isolation

The air-dried entire plant materials of *P. minima* (9.0 kg) were extracted three times with EtOH/H_2O (75 : 25 v/v) (100 L × 2 h). The resulting extract (840 g) was concentrated under a vacuum, suspended in H_2O (5.0 L), and partitioned successively with petroleum ether (3 × 5.0 L), EtOAc (3 × 5.0 L) to generate the EtOAc fraction. The EtOAc fraction (200 g) was subjected to silica gel CC eluted with a gradient of CH_2Cl_2/MeOH (100 : 1 to 0 : 1 v/v) to give eight subfractions (E1–E8). Fraction E3 (40.0 g) yielded four subfractions by silica gel CC eluted with CH_2Cl_2/MeOH (100 : 1 to 0 : 1). Subfraction E33 (15.0 g) was separated on a silica gel CC eluted with petroleum ether/EtOAc (100 : 1 to 0 : 1) to produce five subfractions (E331–E335), subfraction E332 (392.5 mg) was applied to Sephadex LH-20 column eluted with CH_2Cl_2/MeOH (1 : 1) and separated by HPLC (MeOH/H_2O, 70 : 30) to yield **12** (40.0 mg, t_R = 14.0 min). Subfraction E333 (142.3 mg) was separated by HPLC (MeOH/H_2O, 70 : 30) to afford **10** (200.0 mg, t_R = 26.0 min). Subfraction E336 (1.0 g) was purified by HPLC (MeOH/H_2O, 70 : 30) to yield **15** (4.0 mg, t_R = 34.0 min). Subfraction E34 (15.0 g) was subjected to silica gel CC using CH_2Cl_2/MeOH (100 : 1 to 0 : 1) as eluent to produce five subfractions (E341–E345). Subfraction E341 (4.0 g) and E342 (7.0 g) were chromatographed on silica gel column eluted with petroleum ether/acetone (100 : 1 to 0 : 1) and then separated using HPLC (MeOH/H_2O, 70 : 30) to obtain **11** (100.0 mg, t_R = 22.0 min) and **18** (5.0 mg, t_R = 30.0 min). Subfraction E345 (0.62 g) was separated by HPLC (MeOH/H_2O, 70 : 30) to produce **1** (3.0 mg, t_R = 27.0 min). Fraction E4 (30.0 g) was separated by silica gel CC eluted with CH_2Cl_2/MeOH (100 : 1 to 0 : 1) to yield two subfractions (E41–E42). Subfraction E42 (20.0 g) was separated on a silica gel CC eluted with CH_2Cl_2/MeOH (100 : 1 to 0 : 1) to yield two subfractions (E421–E422). Subfraction E421 (12.0 g) and E422 (5.0 g) were chromatographed on a silica gel column eluted with petroleum ether/EtOAc (100 : 1 to 0 : 1) and then separated by HPLC (MeOH/H_2O, 70 : 30) to produce **2** (5.0 mg, t_R = 34.0 min), **8** (20.0 mg, t_R = 39.0 min), and **19** (3.0 mg, t_R = 45.0 min). Fraction E6 (30.0 g) was subjected to silica gel CC eluted with CH_2Cl_2/MeOH (100 : 1 to 0 : 1) to produce five subfractions (E61–E65). Subfraction E64 (15.0 g) was subjected to a silica gel CC eluted with petroleum ether/acetone (100 : 1 to 0 : 1) to produce E641 (1.53 g), Subfraction E641 was applied to a Sephadex LH-20 column eluted with CH_2Cl_2/MeOH (1 : 1), and then purified by HPLC (MeOH/H_2O, 70 : 30) to yield **6** (16.2 mg, t_R = 27.0 min), **9** (30.0 mg, t_R = 25.0 min), and **16** (18.2 mg, t_R = 21.0 min). Fraction E7 (18.0 g) and E8 (13.0 g) were subjected to silica gel CC eluted with CH_2Cl_2/MeOH (100 : 1 to 0 : 1) to produce seven subfractions (E71–E77) and four subfractions (E81–E84), respectively. Subfraction E76 (7.3 g) was separated on a silica gel CC eluted with CH_2Cl_2/acetone (100 : 1 to 0 : 1) to yield three subfractions (E761–E763). Subfraction E762 (2.3 g) was subjected to passage over an ODS silica gel CC eluted with MeOH/H_2O (1 : 9 to 0 : 1) and then purified by HPLC (MeOH/H_2O, 70 : 30) to yield **5** (4.5 mg, t_R = 31.0 min) and **17** (5.0 mg, t_R = 27.0 min). Subfraction E763 (1.0 g) and E77 (1.8 g) were purified by HPLC (MeOH/H_2O, 70 : 30) to yield **7** (3.0 mg, t_R = 25.0 min), **13** (50.0 mg, t_R = 24.0 min), and **14** (42.0 mg, t_R = 26.0 min). Subfraction E83 (2.0 g) was chromatographed on a Sephadex LH-20 column eluted with CH_2Cl_2–MeOH (1 : 1) to yield E832, subfraction E832 (1.1 g) was subjected to ODS silica gel CC, eluted with MeOH/H_2O (1 : 9 to 0 : 1), and then purified by HPLC (MeOH/H_2O, 55 : 45) to yield **3** (25.1 mg, t_R = 23.0 min), **4** (6.7 mg, t_R = 26.0 min).

Physaminilide A (1). White amorphous powder; $[\alpha]_D^{25}$ 45.0 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 206 (3.55) nm; ECD (*c* 4.6 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 249 (+1.75), 289 (−1.27) nm; IR (KBr) ν_{max} 3385, 2918, 1714, 1373, 1234, 1023 cm^{-1}; ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5) data, see Tables 1 and 3; HRESIMS *m*/*z* 543.2583 $[M + H]^+$ (calcd for $C_{30}H_{39}O_9$, 543.2594).

Physaminilide B (2). White amorphous powder; $[\alpha]_D^{25}$ 135.0 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 206 (3.49) nm; ECD (*c* 9.5 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 250 (+2.00), 300 (−0.51) nm; IR (KBr) ν_{max} 3307, 2918, 1710, 1377, 1243, 1021 cm^{-1}; ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5) data, see Tables 1 and 3; HRESIMS *m*/*z* 527.2623 $[M + H]^+$ (calcd for $C_{30}H_{39}O_8$, 527.2645).

Physaminilide C (3). White amorphous powder; $[\alpha]_D^{25}$ −162.0 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 212 (3.77) nm; ECD (*c* 1.0 × 10^{-3} M, MeOH) λ_{max} ($\Delta\varepsilon$) 210 (+7.51), 250 (+4.35), 290 (−3.48) nm; IR (KBr) ν_{max} 3394, 2920, 2840, 1697, 1647, 1468, 1384, 1130 cm^{-1}; ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5) data, see Tables 1 and 3; HRESIMS *m*/*z* 503.2647 $[M + H]^+$ (calcd for $C_{28}H_{39}O_8$, 503.2645).

Physaminilide D (4). White amorphous powder; $[\alpha]_D^{25}$ 45.0 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 205 (3.19), 225 (3.22) nm; ECD (*c* 1.0 × 10^{-3} M, MeOH) λ_{max} ($\Delta\varepsilon$) 251 (+3.73), 290 (−3.31) nm; IR (KBr) ν_{max} 3386, 2918, 1685, 1398, 1140, 1031 cm^{-1}; ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5) data, see Tables 1 and 3; HRESIMS *m*/*z* 505.2800 $[M + H]^+$ (calcd for $C_{28}H_{41}O_8$, 505.2801).

Physaminilide E (5). White amorphous powder; $[\alpha]_D^{25}$ 60.0 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 205 (3.02), 228 (3.11) nm;

ECD (c 9.1 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 253 (+2.51), 289 (−2.00) nm; IR (KBr) ν_{max} 3391, 2913, 1701, 1378, 1255, 1139, 1031 cm^{-1}; ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5) data, see Tables 1 and 3; HRESIMS m/z 547.2933 $[M + H]^+$ (calcd for $C_{30}H_{43}O_9$, 547.2907).

Physaminilide F (6). White amorphous powder; $[\alpha]_D^{25}$ 52.5 (c 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 205 (3.01), 226 (3.11) nm; ECD (c 1.0 × 10^{-3} M, MeOH) λ_{max} ($\Delta\varepsilon$) 249 (+3.33), 292 (−1.97) nm; IR (KBr) ν_{max} 3374, 2915, 1705, 1375, 1224, 1025 cm^{-1}; ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5) data, see Tables 2 and 3; HRESIMS m/z 561.2686 $[M + H]^+$ (calcd for $C_{30}H_{41}O_{10}$, 561.2700).

Physaminilide G (7). White amorphous powder; $[\alpha]_D^{25}$ −38.0 (c 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 218 (3.76) nm; ECD (c 9.2 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 259 (+2.58), 330 (−1.00) nm; IR (KBr) ν_{max} 3394, 3189, 3008, 2920, 2849, 1646, 1468, 1419, 1261, 1119 cm^{-1}; ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5) data, see Tables 2 and 3; HRESIMS m/z 545.2723 $[M + H]^+$ (calcd for $C_{30}H_{41}O_9$, 545.2751).

3-Methoxy-2,3-dihydrowithangulatin A (8). White amorphous powder; $[\alpha]_D^{25}$ 28.0 (c 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 212 (3.78) nm; ECD (c 1.8 × 10^{-3} M, MeOH) λ_{max} ($\Delta\varepsilon$) 250 (+2.81), 290 (−2.36) nm; IR (KBr) ν_{max} 3394, 3189, 2920, 2849, 1646, 1467, 1418, 1253, 1114 cm^{-1}; ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5) data, see Tables 2 and 3; HRESIMS m/z 559.2891 $[M + H]^+$ (calcd for $C_{31}H_{43}O_9$, 559.2907).

3-Methoxy-2,3-dihydrophysagulide D (9). White amorphous powder; $[\alpha]_D^{25}$ 60.0 (c 0.07, MeOH); ECD (c 8.7 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 249 (+2.15), 290 (−2.95) nm; UV (MeOH) λ_{max} (log ε) 203 (3.45) nm; IR (KBr) ν_{max} 3392, 2917, 1809, 1714, 1375, 1236, 1096 cm^{-1}; ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5) data, see Tables 2 and 3; HRESIMS m/z 575.2850 $[M + H]^+$ (calcd for $C_{31}H_{43}O_{10}$, 575.2856).

Cytotoxicity assays

The cytotoxic activity of these compounds for A375 human melanoma cells was determined using an MTT assay.[24] The A375 cells were obtained from ATCC (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) in a humidified atmosphere (37 °C, 5% CO_2). Cells (3 × 10^3 cells per well) were placed in 96-well plates for 24 h. The test compounds with different concentrations were applied to the 96-well plates, incubated for 48 h. 5-Fluorouracil was used as the positive control. Then, MTT (5 mg mL^{-1}) was added to the cells and maintained for 2.5 h. The cells were resolved with DMSO (150 μL) after removed from the medium. All assays were performed in triplicate (Table 4).

Table 4 Cytotoxicity[a] of compounds 1–19 from *Physalis minima*

	IC_{50} (μM)
Compound[b]	A375
2	3.4 ± 0.9
5	2.0 ± 1.1
8	9.4 ± 3.3
10	1.9 ± 0.1
11	1.2 ± 0.4
15	7.3 ± 2.1
5-Fluorouracil[c]	18.5 ± 0.7

[a] Results for the compounds and positive control are expressed as IC_{50} values in μM. [b] Compounds **1**, **3–4**, **6–7**, **9**, **12–14**, and **16–19** were inactive for A375 cells used (IC_{50} > 10 μM). [c] Positive control.

Conclusions

In conclusion, 19 withanolides were isolated from *Physalis minima*, which included eleven previously undescribed withanolides, physaminilide A–G (**1–7**) and two artificial withanolides (**8–9**), together with ten known ones (**10–19**). The structures of the new compounds were elucidated by a combined analysis of the IR, UV, HRESIMS, NMR and electronic circular dichroism (ECD) spectra. All the isolates were assayed cytotoxic activities against human melanoma A375 cells. Compounds **2**, **5**, **8**, **10**, **11** and **15** exhibited significant cytotoxic activities with IC_{50} values in the range of 1.2–9.4 μM.

Conflicts of interest

The authors declare no conflict interest.

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